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INTERACTIONS BETWEEN MOLTING AND REGENERATION IN THE LAND CRAB

CHRISTIE A. HOLLAND[1] AND DOROTHY M. SKINNER

The University of Tennessee — Oak Ridge Graduate School of Biomedical Sciences and the Biology Division, Oak Ridge National Laboratory,[2] Oak Ridge, Tennessee 37830

This laboratory has previously called attention to the efficacy of loss of a critical number of limbs, either pereiopods or chelipeds, in stimulating precocious molts in Crustacea (Skinner and Graham, 1970; 1972). The underlying mechanism for that stimulation is not yet known. The interval between limb loss and ecdysis is somewht longer than that undergone by animals triggered to molt by loss of eyestalks, which contain the neurosecretory X-organ sinus gland complex, the source of an as yet incompletely characterized molt inhibitory hormone (Bliss, 1956; Passano, 1960; Rao, 1965). In most species, animals that have undergone precocious molts initiated by eyestalk removal die at or immediately following ecydsis. By contrast, mortality following limb loss is very low (Skinner and Graham, 1972). In recent years, removing a critical number of limbs has become a common means of inducing precocious molts.

Although the minimal effective stimulus in the land crab, *Gecarcinus lateralis,* is five or more limbs, others have shown that loss of four limbs from the freshwater shrimp, *Palaemonetes kadiakensis,* at all stages of the molt cycle except the late premolt period decreases the duration of the intermolt period by as much as 40% (Stoffel and Hubschman, 1974). Similarly, the number of animals molting within a given time period is directly correlated with the number of walking legs removed from the edible crab, *Cancer pagurus* (Bennett, 1973) and from the fiddler crab, *Uca pugilator* (Fingerman and Fingerman, 1974). The latter authors also showed that in specimens of fiddler crab triggered to undergo precocious molts by loss of eyestalks, subsequent autotomy of walking legs had the converse effect: the duration of the premolt period was significantly *lengthened* in animals from which three or more limbs were autotomized as compared to animals missing eyestalks only or missing eyestalks and one or two limbs.

The present paper describes another set of interactions between regeneration and molting: loss of one or more partially regenerated limbs (primary regenerates) before a critical stage in the premolt period temporarily, for 10 to 14 days, decreases both growth and DNA synthesis of other primary regenerates remaining on the animal while the early stage of re-regeneration is initiated at the sites of the missing limbs. (Throughout the paper, we shall designate as primary regenerates those that form after autotomy of limbs and secondary regenerates those that form after autotomy of primary regenerates.) The duration of the premolt period is also

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lengthened for 10 to 14 days. Under these conditions, stimulation of growth (in the secondary regenerates) and inhibition of growth (in the remaining primary regenerates) are observed simultaneously in the same animal.

The inhibition caused by the loss of regenerates is seen in animals stimulated to undergo precocious molts by either limb or eyestalk removal.

Several preliminary reports of these results have been published (Holland and Skinner, 1974a, b).

Materials and Methods

Animals

Specimens of *Gecarcinus lateralis* were obtained from the Bermuda Biological Station or from J. Van Montfrans or W. Miley, Boca Raton, Florida. Precocious molts were initiated by either removal of both eyestalks or induction of autotomy of eight walking legs (Skinner and Graham, 1970, 1972). Autotomy is the physiological self-amputation of injured limbs at a preformed breakage plane by the abrupt contraction of a set of autotomy muscles in the basi-ischial segment (Wood and Wood, 1932). Animals autotomize limbs when threatened or when the merus segment of the limb is injured. Regeneration of the autotomized limbs occurs early in the premolt period [stage D_0 (Skinner, 1962) and D_1, these data]. The data on the size of the regenerating limb buds are expressed as a regeneration index (R), which is the length of the regenerate (measured at 2.75 × magnification) divided by the maximal width of the carapace × 100 (Bliss, 1956). Because the length of regenerates of ($R \leqq 10$) is difficult to determine precisely, these smallest R values are approximate.

DNA synthesis

For *in vitro* studies, crabs were caused to autotomize regenerating limbs. The regenerates were rinsed several times in sterile 0.5 M NaCl and placed in chambers containing Eagle's Minimal Essential Medium with electrolytes adjusted to concentrations isotonic with *Gecarcinus hemolymph* (Skinner, Marsh and Cook, 1965) and 10% calf serum (Fig. 1). The composition of the medium is listed in Table 1.

Figure 1. Four limb regenerates after autotomy, maintained *in vitro*.

TABLE I

Medium used for maintenance of limb regenerates in vitro.

Powdered MEM	9.51 g
*"100X" Amino acids	30 ml
100X Vitamin	30 ml
100X Glutamine 200 mM	20 ml
10% Na citrate	40 ml
100X Pyruvate (0.1 M)	10 ml
100X Antibiotic mix	10 ml
4 M NaCl	50 ml
0.7 M $CaCl_2$	10 ml
0.06 M $MgSO_4$	100 ml
Glass-distilled H_2O	540 ml
1 M NaOH to adjust pH to 7.3	8.6 ml
H_2O to	900 ml
Fetal calf serum or crab serum, added after filter sterilization to make 10%	100 ml

* Ten milliliters of the "100X" concentrates are normally used for 1000 ml of standard mammalian growth medium. As noted in the right-hand column, we have in some instances used more than this amount for crab tissues. MEM is Eagle's Minimal Essential Medium.

Crystals of phenylthiourea were added to inhibit tyrosinase activity (Harvey and Williams, 1961). Antibiotics were added to final concentrations of 100 units/ml penicillin, 0.25 μg/ml fungizone, and 100 μg/ml streptomycin. ^{3}H-thymidine (specific activity, 20 Ci/mmole) was used at 10–20 μCi/ml culture medium to label DNA. Chambers were rocked at 24 excursions/min at room temperature. The gas phase was air.

DNA was isolated from regenerates by methods described previously (Skinner, 1967) and displayed on two-step "relaxed" preparative neutral CsCl density gradients (Brunk and Leick, 1969). Specific activity of the DNA was determined by monitoring at 260 nm the absorbency of fractions dripped from a hole in the bottom of the gradient tube and by counting aliquots of selected fractions precipitated on Whatman No. 3 filters with 5% TCA (Bollum, 1959). To monitor the uptake of radioactive precursors into cell water, radioactivity in the ethanol-soluble fractions of the DNA preparations was determined by counting aliquots in dioxane.

The crustacean molt cycle

The crustacean molt cycle was divided into four major stages by Drach (1939). We present here a brief description of the molt cycle, of limb regeneration and of their temporal relationship in relatively large specimens of *Gecarcinus*. Stage A immediately follows ecydsis; it is very brief (1 to 2 days). In Stage B, 2 to 5 days, synthesis of endocuticle layers begins and continues into Stage C, during which the exoskeleton hardens further by calcification. The completion of the membranous layer, the innermost layer of the exoskeleton, concludes Stage C_3; Stage C_4, or intermolt period, is the longest part of the cycle, 10 to 11 months in duration, while the premolt period, Stage D, lasts 1 to 2 months. Regeneration of missing limbs occurs only during the premolt period in *Gecarcinus* (Bliss, 1956;

Hodge, 1958; Skinner, 1962) as in many other crustaceans (Emmel, 1910; Bliss, 1960; Adiyodi, 1972).

When a limb is autotomized, a scab is formed by clotted blood and the autotomy membrane that covers the breakage plane (Emmel, 1910; Hodge, 1958). Within two weeks the scab is erupted by a papilla which grows approximately 2 mm and then, in unstimulated (intermolt) animals, enters a state of quiescence, the basal growth plateau, that can last as long as ten months. In 1 mm limb regenerates, tissue differentiation is accompanied by segmentation (Hodge, 1958). Such miniature limbs with clearly defined segments are mobile and can be drawn close to the body. We find that 3-mm long regenerates can be autotomized if stimulated, indicating that functional connections have been re-established to the autotomy muscles located in the basi-ischium of the old limb. During the premolt period, the regenerate grows linearly until one to two weeks before ecdysis when it enters a second growth plateau. At this stage (D_1), the epidermis covering the branchiostegite regions of the carapace separates from the old exoskeleton. The cells enlarge and synthesize the new epi- and exocuticular layers comprising the premolt portion of the exoskeleton (Stage D_2) with which the animal emerges at ecdysis. Formation of exoskeleton on other parts of the body occurs at this time or before, as in the case of epipodites (Tchernigovtzeff, 1959). Since the duration of the premolt period varies from animal to animal, these cytological changes in the epidermis are used for more precise staging. A more complete summary of premolt events that subdivide Stage D into four discrete phases is included in Skinner (1962).

Results

Growth patterns of regenerates of animals with or without eyestalks

The first experiment shows the effect of the presence or absence of eyestalks on the growth pattern of regenerating limbs. In the experiment depicted in Figure 2a, three animals from which eyestalks had been removed one or two days previously and three animals with eyestalks were caused to autotomize eight walking legs on day 0. Regenerating limbs were measured and their R values calculated as described. The curve to the left in Figure 2a shows the pattern of regeneration in animals missing eyestalks; that to the right, in animals with eyestalks.

In animals stimulated to molt by either eyestalk removal alone or by a combination of eyestalk removal plus limb autotomy, an autotomized limb is replaced by a papilla which grows linearly *without* entering the basal growth plateau characteristic of intermolt animals. The pattern of growth of regenerates of animals with eyestalks is similar, though not identical, to that of animals without (Fig. 2a). In both groups of animals blastema formation as well as the growth plateau immediately preceding ecdysis are of approximately 10 to 15 days' duration. The rate of growth of regenerates of animals without eyestalks is more rapid and the preecdysial growth plateau in such animals occurs when the regenerates are smaller ($R = 19$) than in animals induced to molt by limb autotomy ($R = 25$). Correlated with these two factors, the length of the premolt period of animals induced to molt by eyestalk removal is considerably shorter than that of animals induced to molt by limb autotomy (30 to 35 days as compared to 50 to 55 days; Fig. 2a).

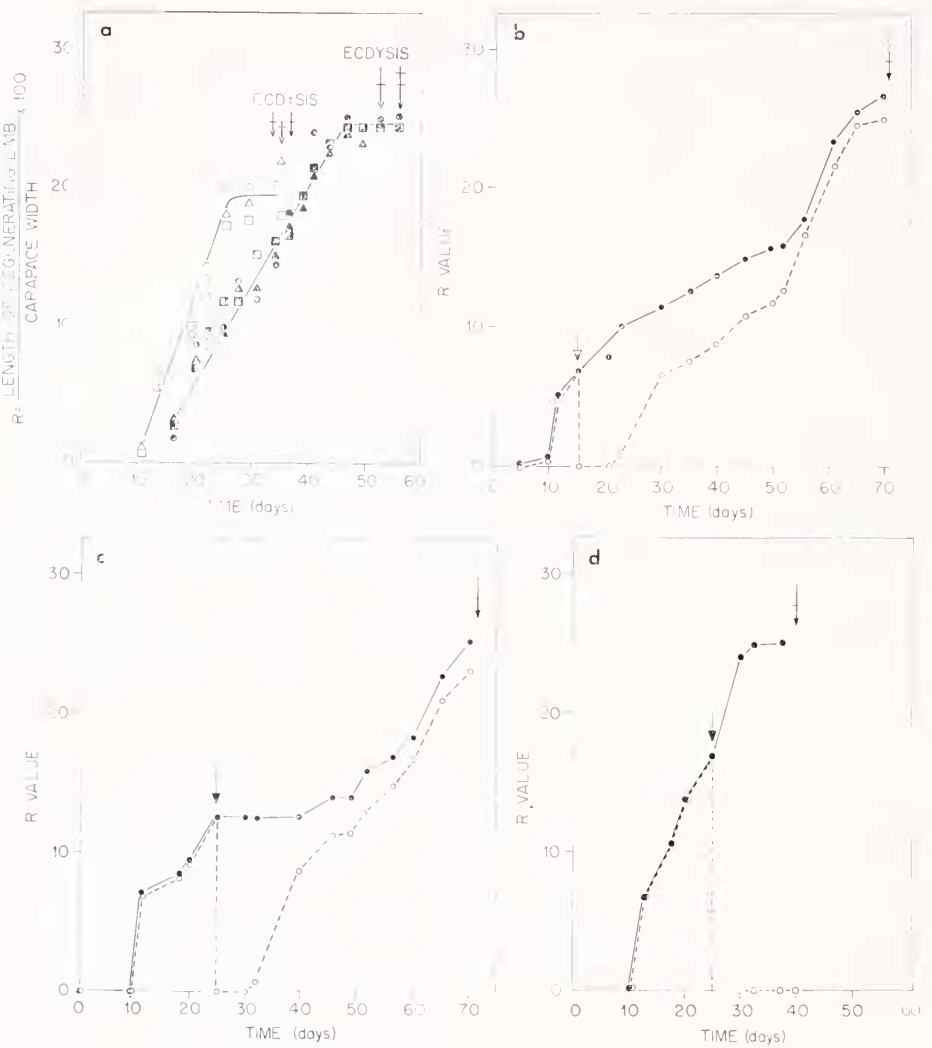

FIGURE 2. Pattern of growth of limb regenerates. Animals were caused to autotomize eight walking legs on day 0. Regenerating limbs were measured and their R values calculated as described in Materials and Methods. (a) R values of regenerates in three animals with (closed triangles, squares and circles) and three animals without (open triangles, squares and circles) eyestalks. Ecdysis of each animal is indicated by a crossed arrow. R values of (b) small, (c) medium, and (d) large regenerates are indicated by closed circles joined by solid lines. The time of autotomy of primary regenerates is indicated by the first arrows. The open circles joined by dotted lines indicate the R values of the secondary regenerates that form following autotomy of primary regenerates.

The growth patterns of primary and secondary regenerates

The effect of loss of partial primary regenerates on the growth of others remaining on the animal at various stages of regeneration has been determined. Animals

were stimulated to undergo precocious molts by autotomy of eight walking legs (Time 0; Fig. 2b, c, d). Four partial regenerates were autotomized (arrow). The growth of one of the partial regenerates remaining *in situ* (primary regenerate, solid lines, Fig. 2b, c, d) was monitored at the same time as was the growth of one of the re-regenerating limbs (secondary regenerate; dashed lines, Fig. 2b, c, d).

The following observations were made. First, subsequent to autotomy of one or more small regenerates ($R = 7$ to 10), the growth rate of the remaining primary regenerates decreases markedly (Fig. 2b). Secondly, following autotomy of larger regenerates, growth of remaining primary regenerates ceases for 7 to 14 days (Fig. 2c). In both cases, a papilla grows from the base of each of the autotomy planes. When the growing secondary regenerates approach the same size as the primary regenerates, the primary regenerates resume growth at a rate similar to that of the secondary regenerates re-establishing the synchronized growth of all regenerates. Just prior to ecdysis the secondary regenerates are slightly smaller than the primary, as are the newly re-regenerated limbs after ecdysis. The merus segment of secondary regenerates is 10 to 15% shorter than that segment in primary regenerates and the weight of secondary regenerates is 10 to 20% lower (data not shown). Autotomy of a fully-formed nonregenerating pereiopod inhibits growth of primary regenerates as in the first and second cases. Third, autotomy of very large regenerates ($R \geqq 20$; stage late D_1 or later), has no effect on the remaining primary regenerates. The autotomized limbs are not regenerated (Fig. 2d) and the animal emerges from ecdysis without its full complement of limbs.

To determine if the inhibition of growth of partial regenerates in *Gecarcinus* was dependent on the autotomy of a critical number of regenerates, as is the initiation of a precocious molting period by limb loss, one primary regenerate was autotomized from three animals and two primary regenerates were autotomized from four other animals, all in stages D_0 to D_1. In every case, growth of the remaining primary regenerates was inhibited while secondary regenerates formed (data not shown). The autotomy of one primary regenerate is as effective as the loss of four or more regenerates in inhibiting growth of other primary regenerates.

Effect of secondary regeneration on the duration of the premolt period

It can be noted in Figure 2 that where growth of primary regenerates slowed (Fig. 2b) or stopped (Fig. 2c) the total premolt period was approximately 70 days; in the case where no inhibition of growth of the primary regenerates occurred (Fig. 2d), ecdysis took place on the forty-second day. A summary of data from a larger population of animals is given in Figure 3. In these experiments, as before, the molting period was initiated by autotomy of eight walking legs (Day 0) and at some later time four primary regenerates were autotomized. Growth of the primary regenerates was monitored and the time of ecdysis noted. The animals in Group 1 were those in which growth of primary regenerates was slowed while secondary regeneration occurred (see Fig. 2b); the animals in Group 2 were those in which growth of primary regenerates stopped while secondary regenerates formed (see Fig. 2c). The animals in Group 3 were those in which growth of primary regenerates was unaffected and there was no secondary regeneration (see Fig. 2d). In Group 3, ecdysis occurred at the same time as in controls (data not

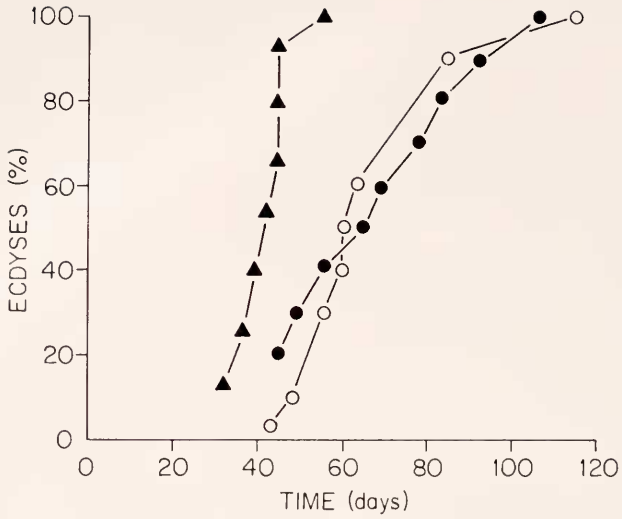

FIGURE 3. Duration of premolt periods of animals with or without re-regenerated limbs. In group 1, 4 of 8 partial regenerates were autotomized from ten animals early in the premolt period ($R = 7$–11). The time at which the animals underwent ecdysis, plotted as percent ecdysis, is indicated by open circles. Primary regenerates were autotomized from animals in Group 2 at $R = 13$–20 (closed circles). Primary regenerates were autotomized from animals in Group 3 in Stage D_2 or later ($R \geqq 20$; closed triangles). All twenty animals in Groups 1 and 2 formed secondary regenerates before ecdysis; those in the third group did not.

shown) from which primary regenerates had not been removed. Groups 1 and 2 in which the autotomy of regenerates was induced about one week earlier than Group 3 were delayed three weeks or longer in the time to ecdysis. It is concluded that the growth inhibition is correlated with a prolongation of the premolt period. This conclusion confirms and extends earlier observations (Skinner and Graham, 1972).

DNA synthesis during limb regeneration

In order to study the DNA synthetic activity of the limb regenerates under controlled conditions, autotomized regenerates were placed *in vitro*. In this way the chemical milieu as well as the specific radioactivity of the ^{3}H-thymidine precursors could be controlled and standardized. ^{3}H-thymidine levels within the tissues were constant, as noted below. The limb regenerates were incubated from 14–16 hr, during which time the rate of labeling remained constant (data not shown).

DNA was isolated and displayed on CsCl density gradients. The characteristic poly d(A-T) satellite and main component DNAs were seen (Skinner, 1967); the G+C-rich satellite was sometimes not detected because of the small amount of DNA centrifuged as well as its low thymidylate content. Some representatives of typical DNA preparations are shown in Figure 4. ^{3}H-Thymidine was incorporated into satellite (arrow) and main component DNAs at the same specific activities

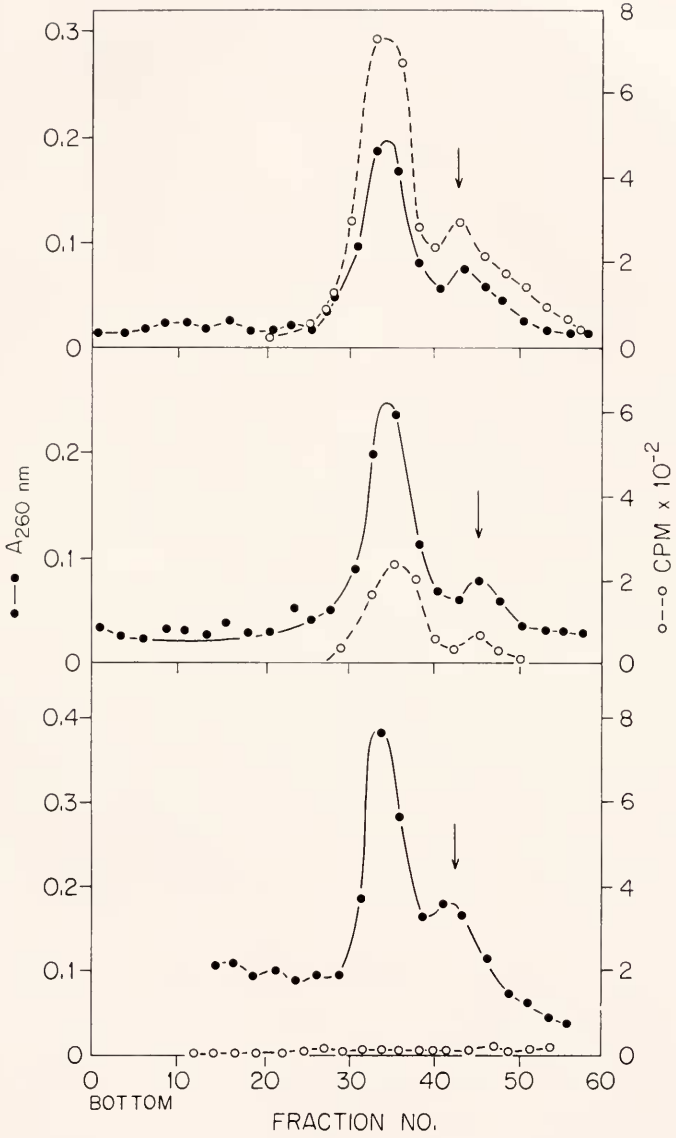

FIGURE 4. Preparative CsCl gradients of DNA from regenerates at different stages of the premolt period. Regenerates were incubated *in vitro* with ^{3}H-thymidine and the DNA was isolated and centrifuged in CsCl gradients. The poly d(A-T) satellite is indicated by the arrow. Fractions were collected, their UV absorbency at 260 nm was monitored, and the TCA-precipitable radioactivity of alternate fractions was determined: (A) early ($R = 7$); (B) middle ($R = 12$); and (C) late ($R = 24$) stages of the premolt cycle. The closed circles and solid line indicate absorbency at 260 nm; open circles and dotted line indicate ^{3}H-thymidine.

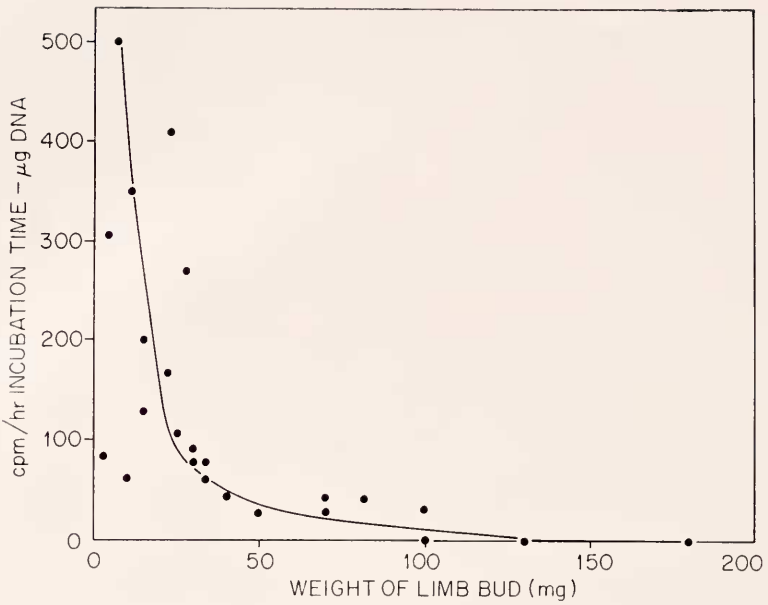

FIGURE 5. Incorporation of ^{3}H-thymidine into DNA during limb regeneration. The specific activity of main component DNA isolated from limb buds of different sizes and treated as described in Figure 3 is plotted versus weight of the regenerate.

when corrected for the different thymidylate contents of each. The DNA in the upper panel was isolated from a set of small, early, primary regenerates ($R = 7$), that in the middle panel from a set of larger regenerates ($R = 12$) taken later in the premolt period and that in the lower panel from large, late regenerates ($R = 24$). The amount of DNA centrifuged was approximately the same in each case. The incorporation of ^{3}H-thymidine is clearly greatest in the earlier regenerates and was indetectable in the large late regenerates. A summary of data from 24 experiments is given in Figure 5. As before, the greatest incorporation (counts/min/μg DNA) occurred in the smallest regenerates (Fig. 5). Incorporation of ^{3}H-thymidine fell to zero in fully formed regenerates (approximately 12 mm in length and 200 mg in a 60-gm animal).

Several weeks before ecdysis (late D_2), the regenerates become pigmented as the epicuticle is synthesized and secreted between the tissue and the sac covering the regenerate. To determine whether the epicuticle that forms on larger regenerates decreases their permeability to radioactive precursors, the radioactivity in cell water was determined by counting the ethanol-soluble fraction of DNA preparations from animals in the early, middle, and late stages of the premolt period. There were insignificant differences in uptake of precursors throughout the period of regeneration (Table II).

DNA synthesis in growing vs. inhibited regenerates

In parallel with the experiments showing that loss of one or more regenerating limbs markedly decreased the growth rate of other primary regenerates, we

TABLE II

Uptake and incorporation of ^{3}H-Thymidine in regenerates.

Stage in premolt period	*R* Value	DNA (cpm × 10^{-3} μg)	Ethanol-soluble radioactivity (cpm × 10^{-5}/g tissue)
Early*	8	3.23	2.80
Middle†	12	1.10	2.52
Late‡	22	0.0009	2.73

* Early = D_0; regeneration of limbs, formation of gastroliths.

† Middle = D_0 to D_1; as above until D_1, separation of epidermis from old exoskeleton (apolysis; Jenkin, 1970).

‡ Late = Animals in stage D_2 (exoskeleton synthesis).

measured the degree of inhibition in the rate of DNA synthesis. In these experiments, autotomized limb buds were used for the zero time controls to establish the level of incorporation of precursors into DNA. The removal of the control limb buds also served as the event leading to the inhibition of growth, and, as will be seen, inhibition of DNA synthesis in remaining regenerates. The remaining regenerates were autotomized 48 hr later and assayed for their rate of DNA synthesis.

In a series of control experiments, the rate of DNA synthesis of four regenerates was compared with the rate of synthesis of the four bilaterally symmetrical regenerates autotomized from the same animals at the same time. As shown in Table III, the rates did not differ by more than 20%.

Four small regenerates were autotomized and their relative rates of DNA synthesis determined *in vitro*. The main band DNA had a specific activity of 4.7×10^3 cpm/μg (Fig. 6a). The four regenerates remaining on the animal, autotomized 48 hr later, had a specific activity of 10 cpm/μg (Fig. 6b). A similar, almost complete cessation of DNA synthesis was seen in nine separate experiments (Table IV-A). In each case, insignificant differences were found in the radioactivity in the cell water, and in each case ecdysis was delayed while secondary regenerates were formed.

To determine whether the inhibition of DNA synthesis induced by loss of partial regenerates was stage-specific, similar experiments were performed with smaller ($R = 8$) and larger ($R = 24$) partial regenerates. A set of four smaller regenerates autotomized on day 0 had a synthetic rate approximately equal to that

TABLE III

Normal variability in DNA synthesis of limb regenerates.

R	SA(a)* (cpm × 10^{-3}/μg DNA)	SA(b)* (cpm × 10^{-3}/μg DNA)	SA(b)/SA(a)
18	2.35	2.18	0.93
14	13.86	12.05	0.87
13	5.58	5.31	0.95
10	6.65	5.59	0.84

* SA(a, b)—specific activity of two groups of regenerates removed on day 0.

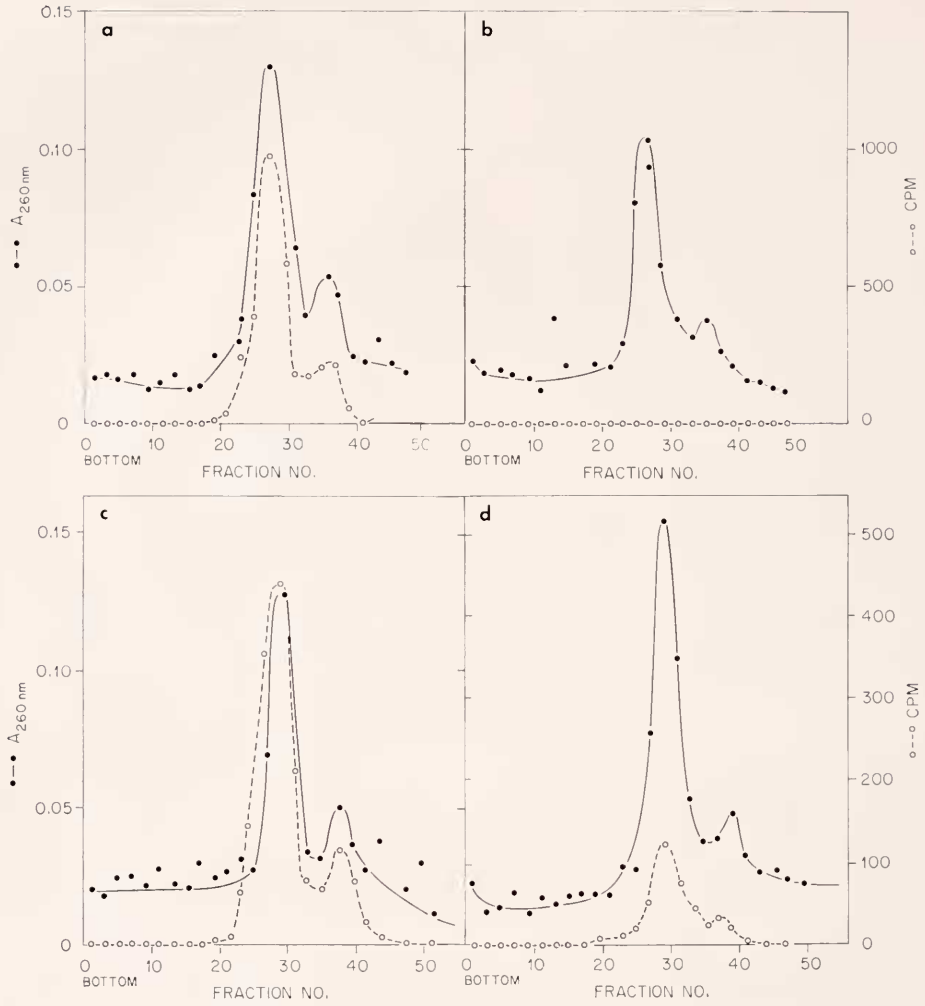

FIGURE 6. Preparative CsCl density gradients of DNA of regenerates from three animals at different stages of regeneration: (a) shows DNA isolated from four regenerates ($R = 14$) incubated with ^{3}H-thymidine as in Figure 4; (b), DNA isolated from bilaterally symmetrical partners to the regenerates in (a) autotomized 48 hr later. The closed circles joined by a solid line indicate absorbency at 260 nm; the open circles and dotted line indicate TCA-precipitable radioactivity; (c) and (d) were the same as (a) and (b) except that primary regenerates were removed early in Stage D_0 ($R = 8$).

shown above (Fig. 6c compared with Fig. 6a). DNA synthesis in their bilaterally symmetrical partners removed 48 hr later was approximately 25% the rate of those removed at time 0 (Fig. 6c and 6d; Table IV-B). This pattern of deceased rather than completely inhibited DNA synthesis corresponds to the observed decrease in the rate of growth of regenerates of similar size (*cf.* Fig. 2b). Larger primary regenerates ($R = 24$), autotomized on day 0, had approximately the same

TABLE IV

The effect of autotomy on DNA synthesis in remaining primary regenerates.

R†	SA of first set of regenerates SA(a)‡	SA of second set of regenerates SA(b)§	SA(b)/SA(a)
(A) Growth of primary regenerates stopped			
20	4.90	0.41	0.084
18	10.39	0.60	0.058
17	3.23	0.06	0.019
17	6.12	0.02	0.003
16	1.74	0.06	0.035
16	2.57	0.12	0.047
15	6.97	0.05	0.007
15	1.09	0.09	0.083
14	4.62	0.06	0.013
*13	14.05	0.86	0.061
*9	28.48	1.12	0.039
(B) Growth of primary regenerates decreased			
14	3.16	0.67	0.212
*13	10.13	2.07	0.204
12	7.28	2.04	0.280
*12	19.98	6.21	0.311
*12	3.44	1.89	0.550
9	6.04	0.56	0.093
8	1.56	0.47	0.301
(C) Growth of primary regenerates not affected			
24	0.15	0.18	1.20
20	0.70	0.69	0.99
18	1.40	1.41	1.01
*15	2.23	2.40	1.08
*14	4.90	5.09	1.04

* Animals without eyestalks.

† R = R value (see Materials and Methods).

‡ $SA(a)$ = specific activity (cpm $\times 10^{-3}/\mu g$) DNA isolated from regenerates which had been removed on day 0, and then incubated *in vitro* 16 hr with ^{3}H-thymidine.

§ $SA(b)$ = *as* SA(a), except regenerates removed on day 2.

rate of DNA synthesis as those removed from the same animal 48 hr later (data not shown). This corresponds to the data in Figure 2d which show that the growth of large primary regenerates is not inhibited by loss of their partners.

In several control experiments, both rates of DNA synthesis and growth patterns were determined on the same animals (data not shown). In one typical experiment, we found that three small regenerating limbs had a rapid synthetic rate; their three bilaterally symmetrical partners, autotomized 48 hr later, synthesized DNA at a decreased rate. Two regenerates were not removed; their rate of growth, followed for the ensuing ten days, decreased as had that of the primary regenerates

of similar size previously described (Fig. 2b). Thus it was possible to observe in a single animal parallel inhibition of both growth and DNA synthesis. Again, re-regeneration of the missing legs occurred during the induced premolt pause.

Neither the rate of DNA synthesis (Table IV-C) nor the growth pattern (Fig. 2d) of very large regenerates was affected by the removal of one or more partial regenerates. These animals had apparently passed the critical period after which ecdysis can no longer be delayed (Skinner and Graham, 1972).

The differences between the rates of DNA synthesis of the partial regenerates removed on day 0 and those removed 48 hr later cannot be attributed to the normal size-associated decrease shown in Figure 5. In all experiments described, the size of the regenerates removed on day 0 was very similar to that of those removed 48 hr later. The time required for DNA synthesis to fall by 50% as a function of normal growth as in Figure 5 is on the order of a week. For DNA synthesis to be affected by as much as has been observed in Group A of Table IV requires more than a month if the regeneration is undisturbed. It is concluded that the decreased synthesis described in Table IV is not a function of the very small amount of growth that might occur in the 48 hr time interval between the two samples.

DNA synthesis in regenerates of animals without eyestalks

The data described thus far could be accounted for by the minimal hypothesis that the inhibition of growth and DNA synthesis after autotomy of partial regenerates is induced by secretion of the molt-inhibitory hormone, synthesized and stored in the crustacean eyestalk. To test this, experiments were performed on animals from which eyestalks had been removed. Data from regenerates of animals without eyestalks whose growth pattern was similar to that of regenerates of animals with eyestalks are indicated by an asterisk in Table IV. Growth and DNA synthesis of primary regenerates remaining after autotomy of their partners showed stage-dependent inhibition comparable to that observed in intact animals still capable of producing the molt inhibitory hormone.

In animals without eyestalks, the autotomy of slightly smaller partial regenerates ($R = 7$–10) produces complete inhibition of DNA synthesis equivalent to that caused by the loss of larger regenerates in animals with eyestalks. This smaller R value is comparable to that of animals whose DNA is depicted in Figures 6a and b since, as noted in the section *Growth of regenerates of animals with or without eyestalks,* at ecdysis primary regenerates of animals without eyestalks are smaller than those of animals with eystalks.

These experiments show that DNA synthesis and growth can be effectively blocked in the absence of molt-inhibitory hormone, indicating that an additional physiological inhibitory factor(s), not necessarily hormonal, is functional in these animals.

Discussion

The coarse controls on molting are thought to be the interaction of two hormones: a molt inhibitory hormone (MIH), of the X-organ sinus gland complex,

which predominates during the intermolt period; and a growth-promoting hormone, probably ecdysterone, that stimulates growth when present in a favorable concentration with respect to the antagonistic MIH. Although there must be many fine controls on the growth and differentiation of various tissues in the whole animal, the interaction of these two hormones has been thought to be primary in regulating "growth" *vs.* "non-growth". This simple picture is complicated by the present experiments in which growth inhibition and growth stimulation are simultaneously demonstrable in a single animal. In addition to the data described in the results above, apolysis and synthesis of a new exoskeleton are considerably delayed in animals whose premolt growth has been slowed by autotomy of partial regenerates (unpublished data).

Before discussing the present work on the limb regenerates and its relation to the work of others, the evidence for the functioning of MIH and ecdysterone or an ecdysterone-like hormone in Crustacea will be considered briefly. The role of MIH was suggested by experiments in which extirpation of X-organ or the X-organ sinus gland complex was effective in triggering molting (Passano, 1953). Rao (1965) isolated from the eyestalks of the crab, *Ocypode macrocera*, and partially characterized a peptide that delayed ecdysis when injected into eyestalkless animals. The role of the Y-organs was suggested by the fact that molting is prevented by the removal of Y-organs from the shore crab, *Carcinus maenas* (Echalier, 1954, 1959) and similarly, that after the Y-organ has been removed regenerates do not grow beyond basal plateau in the marsh crab, *Sesarma reticulatum* (Passano and Jyssum, 1963) and from the crab, *Pachygrapsus marmoratus* (Charmantier-Daures and Vernet, 1974). In a further set of experiments, the latter authors not only removed Y-organs but also eight pereiopods. In that case, 13 of 60 animals completed preparations for molt including complete regeneration of the missing limbs. Unfortunately, the authors did not indicate whether the animals, all of which died before ecdysis, were subjected to histological examination to determine if the Y-organs had been completely removed.

The possibility that ecdysterone is the active substance produced by the Y-organs was suggested by the isolation from green crabs of a substance that caused pupation of the fly, *Calliphora*, and was presumably ecdysterone (Karlson and Skinner, 1960). In addition, Horn and colleagues have isolated from green crabs a steroid with chromatographic and spectral properties similar to those of ecdysone isolated from insects (Hampshire and Horn, 1966; Horn, Middleton and Wunderlich, 1966). Commercially available ecdysterone is substantially less effective in Crustacea than in insects (Skinner and Graham, 1970) and may be highly toxic at doses that do not initiate molting (Lowe, Horn and Galbraith, 1968). The latter workers found that at lower doses, ecdysterone decreases the duration of the premolt period of crayfish that have already been stimulated to molt by eyestalk removal. Krishnakumaran and Schneiderman (1968) found it ineffective except at very high—although not pathological—levels in the amphipod, *Armadillidium*.

It is worth noting that although premolt growth of regenerates in crabs might require ecdysterone or an ecdysterone-like growth hormone, papilla formation and basal growth of regenerates do not show such dependence and occur in the absence of Y-organs (Passano and Jyssum, 1963) or even during intermolt when MIH is thought to predominate (Bliss, 1956; Hodge, 1958; Skinner, 1962).

The present paper is a continuation of earlier studies (Skinner and Graham, 1970, 1972) showing that the loss of more than four appendages is an adequate stimulus to molting preparations in intermolt specimens of *Gecarcinus*. This paper reports that the autotomy of only one (or more) of the ensuing primary regenerates may be effective in inhibiting further preparations for molt, *i.e.*, the continued growth of the remaining regenerates or exoskeleton formation; during the period of inhibition a second blastema forms at the autotomy plane of each missing primary regenerate. The magnitude of the inhibitory effect depends on the stage of the animal in its molting preparations. Prior to a certain critical time (late D_0 or early D_1), the growth of the remaining primary regenerates is slowed or stopped while the secondary regenerates "catch up". After the critical time (late D_1 to ecdysis) autotomy of partial regenerates does not influence the remaining regenerates or the duration of the molting period. It has long been known that limb regeneration in Crustacea is coordinated with molting. The second autotomy breaks the close tie between these growth events. The phenomena described here have the effect of re-synchronizing the experimentally dissociated events of early premolt. Since regeneration is limited to this period and cannot occur during the long intermolt period, the biological adaptiveness of this re-synchrony is clear.

A similar interaction between the reactive (regenerating) tissue and the controlling system has been suggested in insects, where the controlling system is thought to be hormonal. Regeneration of an appendage or its imaginal disc delays ecdysis (O'Farrell and Stock, 1953, 1954; Stock and O'Farrell, 1954; Pohley, 1965; Madhavan and Schneiderman, 1969). Further, ecdysterone has been shown to be necessary for the regeneration of imaginal discs (Madhavan and Schneiderman, 1969) and has been implicated in the acceleration of DNA synthesis accompanying growth in insects (Krishnakumaran, Berry, Oberlander and Schneiderman, 1967; Oberlander, 1969).

Pohley (1961) found that the number of mitoses in a normal imaginal disc was decreased while a regenerate was being produced and postulated that the depletion or lack of a stimulatory substance (ecdysterone) could cause the inhibition of growth. Since the early phases of crustacean limb regeneration do not require the presence of ecdysterone (Passano and Jyssum, 1963), it is unlikely that the inhibition reported here could be caused by secondary regenerates selectively sequestering ecdysterone. Therefore, to account for the inhibition of premolt growth of primary regenerates by depletion of a stimulatory factor, a second factor would be required, such as an enzyme that specifically destroys ecdysterone. There is precedent for this suggestion. Between day 4 and 5 of the last larval instar in the hornworm, *Manduca sexta*, juvenile hormone rapidly disappears (Nijhout and Williams, 1974). A hormone-specific esterase has been postulated as the causative agent (Sanburg, Kramer, Kézly and Law, 1975).

The nature of the mechanisms controlling the events reported in this paper is not known. If the signal is hormonal, autotomy could cause the release of a blood-borne inhibitor. The inhibition of both growth and DNA synthesis in regenerates of animals without eyestalks, missing the putative source of MIH, leads to the conclusion that either another inhibitor is responsible or MIH can be produced (or stored and its release triggered by limb loss) elsewhere in the body.

In order to postulate that the controlling factor is neural, one must accept the constraint that the physiological response is determined to a large part by the target tissue. This conclusion is based on the following observations. First, assuming that the innervation of fully formed limbs is the same as that of their regenerates, cutting the *same* nerves stimulates preparations for a precocious ecdysis in one instance and inhibits premolt growth of primary regenerates and further preparations (other than the initiation of blastema formation of secondary regenerates) in a second. In both cases, the initial effect of the loss of five or more limbs or one or more partial regenerates is to cause a new cycle of regeneration of the lost limbs. Secondly, different *numbers* of nerves are involved in the two phenomena. The stimulation of precocious molts requires the loss of five or more limbs in *Gecarcinus*, the loss of only one partial regenerate inhibits growth and DNA synthesis in the remaining regenerates.

The long duration of the effect argues against a single nervous stimulus unless there are further consequences. In a possibly analogous case the bug, *Rhodnius prolixus,* undergoes molting preparations following the stimulation of the abdominal ganglia due to the distension of the body wall after a blood meal (Wigglesworth, 1934). It is thought that the nervous stimulus triggers the release of the prothoracicotropic hormone which, in turn, provokes the release of molting hormone (Wigglesworth, 1970).

The present demonstration of simultaneous inhibitory and stimulatory effects caused by the loss of primary regenerates emphasizes the fine and multifaceted control of the crustacean molt cycle.

Note Added In Press

While this paper was in review, our attention was called to a publication by Tchernigovtzeff (1974). In general, that paper corroborates the results presented in this paper and our earlier reports (Holland and Skinner, 1974a, b). An apparent discrepancy is that in some cases Tchernigovtzeff failed to observe inhibition of growth of primary regenerates following the loss of a fully formed limb from animals missing eyestalks. The reasons for this discrepancy are not clear; we invariably found inhibition of both growth and DNA synthesis in all early premolt animals observed. The experimental protocols differed in several respects. In any case, Tchernigovtzeff's results and ours agree in demonstrating that growth inhibition and growth stimulation can occur simultaneously in individual animals. We conclude from these observations that there are finer controls than simply the ratio of two hormones in the general circulation.

Summary

1. Following the loss of one or more primary regenerates from the land crab, *Gecarcinus lateralis,* before a critical stage of the molt cycle (probably D_1, early), secondary regenerates form to replace those lost.

2. Although loss of at least five limbs from an intermolt animal is required to initiate molting preparations (Skinner and Graham, 1972) loss of only one primary regenerate, prior to the critical time, is sufficient to inhibit these preparations.

3. Following the loss of one or more primary regenerates before a critical time, the rate of growth and the synthesis of DNA in other primary regenerates that remain *in situ* decrease or cease temporarily (10 to 14 days).

4. The precise stage of the animal in the molt cycle determines whether growth and/or DNA synthesis decrease or cease in the remaining primary regenerates. Loss of regenerates early in D_0 ($R = 7$–10) causes a decrease; loss of regenerates later in D_0 or early D_1 ($R = 10$–17) causes complete inhibition.

5. The duration of the premolt period is lengthened by the same time as that during which the early stages of re-regeneration of the missing regenerates occur.

6. Primary regenerates removed after the critical point in the premolt period are not re-regenerated before ecdysis; the premolt period is not extended and the animal emerges without a full complement of limbs.

7. Primary regenerates in animals without eyestalks respond similarly to those in animals with eyestalks. This excludes the possibility that the inhibition is caused by release of MIH from the sinus gland-X organ complex. Other physiological factors must be posulated.

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